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Ruthenium Red inhibits the activation of pyruvate dehydrogenase caused by positive inotropic agents in the perfused rat heart

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1. The increases in the amount of active, non-phosphorylated, pyruvate dehydrogenase caused by positive inotropic agents (from a control value of about 10%, to 40% of total enzyme) in the perfused rat heart could be completely blocked by prior perfusion with 2.5 μg of Ruthenium Red/ml. A similar increase caused by 5 mM-pyruvate was not blocked. 2. This concentration of Ruthenium Red caused a 25% decrease in contractile force of hearts perfused in the absence of positive inotropic agents; however, in their presence the contractile force reached the same value in the absence or presence of Ruthenium Red. 3. Neither control nor stimulated phosphorylase *a* content was affected by Ruthenium Red. 4. Verapamil (0.1 μM) also decreased control contraction (by 40%), but did not block the activation of pyruvate dehydrogenase caused by a rise in extracellular [Ca^{2+}]. 5. The results support the hypothesis that positive inotropic agents activate pyruvate dehydrogenase in rat heart by increasing intramitochondrial [Ca^{2+}].

It is well established that positive inotropic agents influence contraction by increasing cytoplasmic [Ca^{2+}] in heart cells (see Williamson, 1975; Marban *et al.*, 1980). These agents also cause an acute increase in the amount of PDH_a (from about 10 to 40% of total enzyme) in perfused hearts from fed rats (Hiraoka *et al.*, 1980; McCormack & Denton, 1981). These reports proposed that this was a consequence of increased cytoplasmic [Ca^{2+}] in systole (perhaps 'time-averaged') leading to an increase in intramitochondrial [Ca^{2+}] and hence activation of pyruvate dehydrogenase phosphate phosphatase, which is Ca^{2+}-sensitive (Denton *et al.*, 1972). This concept was supported by the observation that an increase in extramitochondrial-medium [Ca^{2+}] within the expected physiological range caused an increase in PDH_a content in isolated rat heart mitochondria (Denton *et al.*, 1980). Also, McCormack *et al.* (1982) found that the changes in the amount of PDH_a in response to positive inotropic agents in the perfused rat heart under several different conditions (e.g. starvation, diabetes, the absence or presence of pyruvate) were closely matched by the changes in the amount of PDH_a in response to extramitochondrial Ca^{2+} in isolated mitochondria. Furthermore, Hiraoka *et al.* (1980) demonstrated that the whole-tissue concentrations of the metabolites which are the other known effectors of the pyruvate dehydrogenase system (e.g. ATP, NADH, NAD, acetyl-CoA, CoA) were not appreciably altered by β-adrenergic agonists.

RR is a hexavalent polysaccharide stain (Luft, 1971) which inhibits the uptake of Ca^{2+} ions into isolated mitochondria with a high degree of specificity (Moore, 1971; Vasington *et al.*, 1972). It also inhibits the increase in PDH_a content of isolated mitochondria in response to an increase in extramitochondrial [Ca^{2+}] (Denton *et al.*, 1980). We have therefore examined the effects of RR on the activation of pyruvate dehydrogenase caused by positive inotropic agents in the perfused rat heart.

Abbreviations used: PDH_a, the active, non-phosphorylated, form of pyruvate dehydrogenase; RR, Ruthenium Red.

Experimental

Hearts from male Wistar rats (220–280 g) were perfused by the Langendorff technique (drip-through without recirculation) at 37°C, with gassed (O_2/CO_2, 19:1) bicarbonate-buffered medium (after Krebs & Henseleit, 1932). Unless otherwise stated in Figure and Table legends, this contained 1.5 mM-$CaCl_2$, 0.25 mM-EGTA, 0.2 mM-KH_2PO_4 and 10 mM-glucose; the perfusion pressure was 7 kPa. At the end of the perfusions the hearts were freeze-clamped.

Frozen tissue was extracted and assayed for both the amount of PDH_a and the total amount of

pyruvate dehydrogenase present as described by Whitehouse *et al.* (1974). Extraction of frozen tissue and assays for both the amount of phosphorylase *a* and the total amount of phosphorylase present were as given by England (1976). Results for both enzymes are given as percentages of the total enzyme existing in the active form. The total activities of both enzymes were unaffected by any of the conditions tested. Contractile force at the peak of the length–tension curve was monitored as described by England (1976).

All chemicals and substrates were obtained from either Boehringer Corp., Lewes, East Sussex, U.K., or BDH Chemicals, Poole, Dorset, U.K., except for isoprenaline (Kodak, Kirkby, Liverpool, U.K.), verapamil (Knoll A.G., 4410 Liestal, Switzerland) and RR (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.). The amounts of RR given (μg/ml) refer to the compound as provided; this was stated to have a dye content of 45% (2.5 μg of RR/ml is approx. 3 μM).

Results and discussion

The hypothesis detailed in the introduction for the mechanism by which positive inotropic agents increase the amount of PDH_a in the perfused rat heart was supported by preliminary experiments which showed that the increase could be blocked by the presence of 10 μg of RR/ml of medium. However, although the RR did not prevent the stimulation of contraction caused by inotropic agonists, it was clear by visual inspection that before agonist addition the hearts perfused with RR were beating less forcibly than controls. This raised the possibility that RR caused a general decrease in cytoplasmic $[Ca^{2+}]$ and that the block of the increase in PDH_a was due to this, rather than its blocking of mitochondrial Ca^{2+} uptake. Therefore it was decided also to monitor contraction and phosphorylase *a* content as indicators of cytoplasmic $[Ca^{2+}]$. It was also decided to perform most of the experiments by using an increase in extracellular $[Ca^{2+}]$ as the inotropic stimulus. This avoided the increases in [cyclic AMP] that would result from adrenergic agonists, as these would lead to an activation of phosphorylase kinase independently of Ca^{2+} (see Cohen, 1978).

A minimum of 2.5 μg of RR/ml of medium was found to be able to block the increases in PDH_a caused by positive inotropic agents (see Fig. 2*b*). This concentration was used to study the effects of RR on the time courses of changes in contraction and the contents of PDH_a and phosphorylase *a* in the perfused rat heart after a rise in extracellular $[Ca^{2+}]$ to 6 mM (Fig. 1). In control perfused hearts the force of contraction gradually decreased by about 8% over a 5 min period after a 5 min preperfusion (Fig. 1*a*, zero time), presumably owing to tissue oedema. The addition of 2.5 μg of RR/ml after the 5 min preperfusion caused a rapid decrease of about 25–30% in contractile force (half-time, $t_{\frac{1}{2}} \simeq 30$ s; complete by about 2 min), followed by a gradual

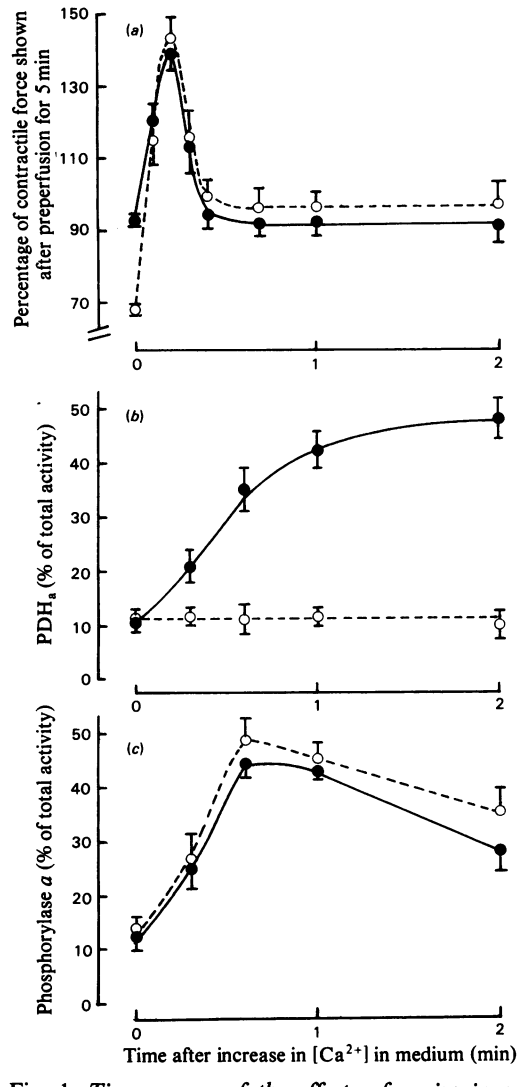

Fig. 1. *Time-courses of the effects of a rise in extracellular* $[Ca^{2+}]$ *on (a) contractile force, (b)* PDH_a *content and (c) phosphorylase a content of rat hearts perfused in the absence or presence of Ruthenium Red*

Hearts were either perperfused with control medium (see the Experimental section) for 10 min (●) or with control medium for 5 min followed by 5 min with the additional presence of 2.5 μg of RR/ml (○). Similar results were obtained if the RR was present for the whole 10 min preperfusion period. In each case (with or without RR), $[Ca^{2+}]$ in the medium was raised to 6 mM at zero time. Results are given as means ± S.E.M. (bars) for at least three hearts at each point.

Table 1. *Effects of pyruvate, adrenaline, isoprenaline, raised* $[Ca^{2+}]$ *in the medium and higher perfusion pressure on the amount of* PDH_a *in rat hearts perfused with no additions, Ruthenium Red or verapamil*

Hearts were preperfused at 7kPa with either control medium (see the Experimental section) for 10min or with control medium for 5min followed by 5min with the additional presence of either 2.5μg of RR/ml or 0.1μM-verapamil, except that, where indicated, 5mM-pyruvate was also present for the whole 10min period (similar results were obtained if RR was present for the 10min preperfusion, with pyruvate being added after 5min). Perfusions were then continued for 1min with further additions of $CaCl_2$ or adrenergic agents, or a rise in pressure, as indicated. Results are given as means ± S.E.M. for the numbers of perfusions in parentheses. $*P \leqslant 0.05$ and $**P \leqslant 0.001$ for the effect of RR or verapamil compared with the appropriate 'no addition' value (i.e. across rows), and $\dagger P \leqslant 0.05$, $\dagger\dagger P \leqslant 0.01$ and $\dagger\dagger\dagger P \leqslant 0.001$ for the effect of addition or change (indicated in left-hand column) compared with the appropriate 'None' value (i.e. down columns), by Student's *t* test.

Additions or changes to preperfusion and/or perfusion medium	PDH_a (as % of total activity) in hearts preperfused for 5min and perfused for 1min with:		
	No additions	2.5μg of RR/ml	0.1μM-verapamil
None	10.1 ± 1.1 (12)	10.9 ± 1.0 (9)	9.1 ± 1.5 (5)
5mM-Pyruvate	34.0 ± 2.3 (5)†††	32.7 ± 2.5 (5)†††	—
$[Ca^{2+}]$ in medium raised to 6mM	42.2 ± 2.6 (10)†††	12.7 ± 1.2 (10)**	38.9 ± 3.5 (3)†††
1μM-Adrenaline	40.5 ± 3.6 (4)†††	11.9 ± 1.1 (4)**	—
0.2μM-DL-Isoprenaline	46.0 ± 3.2 (4)†††	9.0 ± 2.6 (4)**	20.3 ± 3.0 (3)*††
Perfusion pressure increased to 16kPa	35.6 ± 4.7 (3)†	13.4 ± 2.1 (3)*	—

decrease parallel to that of controls, so that just before the stimulus was given the contractile force was about 75% of control (Fig. 1*a*, zero time). A similar resultant contractile force was obtained if the RR was present for the whole 10min preperfusion. This time course for the effect of RR on contractile force in control perfusions (results not shown) was similar at all concentrations tested (up to 10μg/ml), even though the magnitude of the effect was concentration-dependent (Fig. 2*a*). RR had no effect on the frequency of beating, only on the force of contraction.

Fig. 1(*a*) shows that, although RR decreases control contractile force, following the rise in $[Ca^{2+}]$ in the medium the force of contraction in the presence of RR becomes coincident with that in its absence, both at peak contraction (after 10s) and thereafter. This suggests that after stimulation the cytoplasmic $[Ca^{2+}]$ is similar in both conditions. This contrasts with the clear inhibition by RR of the rise in PDH_a content after the rise in $[Ca^{2+}]$ in the medium (Fig. 1*b*). Furthermore, both the control phosphorylase *a* content, and the rise as a result of an increased cytoplasmic $[Ca^{2+}]$ in the stimulated condition, are unaffected by RR (Fig. 1*c*).

Results obtained with 0.2μM-DL-isoprenaline or 1μM-adrenaline as the inotropic stimulus gave very similar results (in both the absence and the presence of RR) to those shown in Fig. 1, except that phosphorylase *a* content rose to about 85% of the total enzyme, presumably because these agents also increase [cyclic AMP] (not shown in full; see Fig. 2 and Table 1). An increased work load, which increases cytoplasmic $[Ca^{2+}]$ but not [cyclic AMP] (see Williamson, 1975), gave results similar to those obtained on raising $[Ca^{2+}]$ in the medium (not shown in full; see Table 1). These results and those of Fig. 1 strongly suggest that the addition of RR to an intact heart preparation does not significantly affect the rise in cytoplasmic $[Ca^{2+}]$ that occurs during inotropic stimulation, but does inhibit the uptake of Ca^{2+} ions into mitochondria, which is the normal consequence of such a rise in cytoplasmic $[Ca^{2+}]$.

This conclusion is supported by the data of Fig. 2, where the effects of increasing [RR] in the medium (up to 10μg/ml), under both control and stimulated (by isoprenaline or a rise in $[Ca^{2+}]$ in the medium) conditions, on the same parameters as in Fig. 1 are shown. Fig. 2(*a*) shows that it is only contractile force in the absence of positive inotropic agents which is affected by RR, whereas in their presence both the peak (after ~10s) and the resultant (after 1min) contractile force are unaffected. This contrasts with PDH_a content (Fig. 2*b*), where the control is unaffected but the stimulated value is markedly inhibited, until by 2.5μg of RR/ml the positive inotropic agents no longer increase PDH_a content. This is much more pronounced than the inhibition of control contraction, suggesting a higher degree of sensitivity to RR of the mitochondrial system. Fig. 2(*c*) shows that phosphorylase *a* content is again unaffected by RR under both control and stimulated conditions.

RR appears to decrease the contraction of control perfused hearts in a manner which is characteristic of the slow Ca^{2+}-channel blockers (see Fleckenstein, 1977), and it remained possible that it blocked the pyruvate dehydrogenase activation as a result of this action rather than an inhibition of mitochondrial Ca^{2+} uptake. We therefore examined the effects

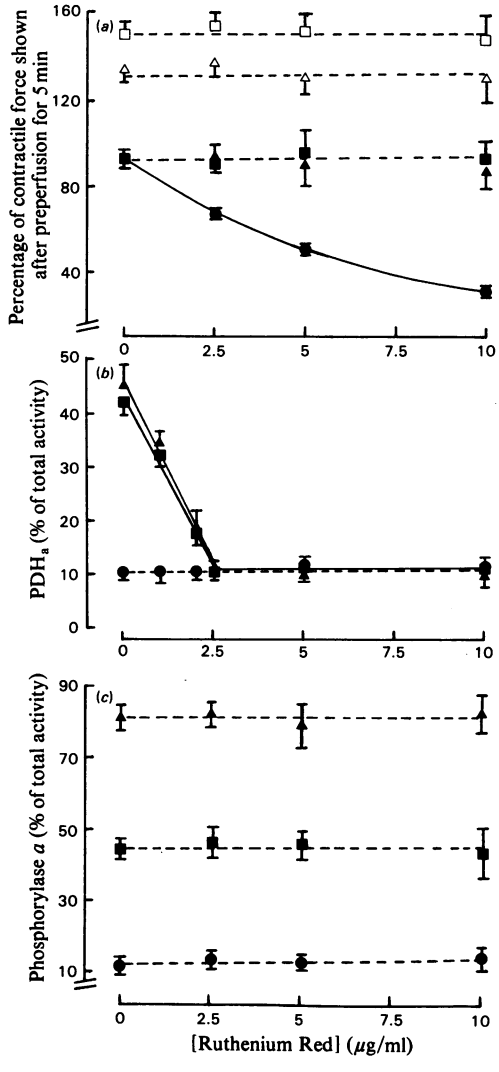

Fig. 2. *Effects of increasing [Ruthenium Red] in the medium on (a) contractile force, (b) PDH_a content and (c) phosphorylase a content of rat hearts perfused with no additions, isoprenaline, or a rise in extracellular $[Ca^{2+}]$*

Hearts were preperfused for 5 min with control medium (see the Experimental section) and then for a further 5 min with the additional presence of the concentrations of RR shown. At each [RR], perfusions were then continued for either 10 s (△, □) (i.e. peak contraction) or 1 min (●, ▲, ■) with no additions (●), 0.2 μM-DL-isoprenaline (△, ▲), or with $[Ca^{2+}]$ in the medium raised to 6 mM (□, ■). Results are given as means ± S.E.M. (bars) for at least three hearts at each point. Parameters that appear to be unchanged by RR are shown by broken lines.

of the well-characterized slow Ca^{2+}-channel blocker, verapamil (see Fleckenstein, 1977), in similar experiments to those described above for RR. Added after a 5 min preperfusion, 0.1 μM-verapamil decreased the force of contraction to about 60% of control; however, unlike that by RR, this decrease was more gradual and appeared to be still occurring when the positive inotropic agents were administered after a further 5 min. In this instance, when $[Ca^{2+}]$ in the medium is raised, both contraction and phosphorylase *a* content rise in a similar manner to that shown in Figs. 1(*a*) and 1(*c*) respectively. However, in the presence of verapamil the increase in PDH_a content was not inhibited (Table 1), in contrast with Fig. 1(*b*), where RR was present. Verapamil did decrease the response of PDH_a to isoprenaline (as was found also by Hiraoka *et al.*, 1980) (Table 1). However, in this instance both the contractile response and phosphorylase *a* response were also diminished (by about 30%) with respect to control responses, presumably because full activation of the slow Ca^{2+} channels, and hence maximal cytosolic $[Ca^{2+}]$, cannot be achieved (see Fleckenstein, 1977) (results not shown).

Another possible explanation for the effects of RR on the activation of pyruvate dehydrogenase was that in some way it inhibited the dephosphorylation of the enzyme other than by preventing Ca^{2+} entry into mitochondria and subsequent phosphatase activation. However, in Table 1 it is shown that RR does not affect the increase in PDH_a caused by the presence of 5 mM-pyruvate in the perfusion medium (Kerbey *et al.*, 1976). This is of similar magnitude to that caused by positive inotropic agents and is presumably due to pyruvate inhibition of PDH_a kinase (see Kerbey *et al.*, 1976).

General conclusions

The results of this study suggest that RR can enter heart cells and inhibit mitochondrial Ca^{2+} uptake *in situ*. The data obtained therefore support the hypothesis that positive inotropic agents activate rat heart pyruvate dehydrogenase by increasing intramitochondrial $[Ca^{2+}]$. It is hoped to exploit this property of RR in future studies to assess the importance of this probable increase in intramitochondrial $[Ca^{2+}]$ in the response of the heart to positive inotropic agents. There are two other enzymes which occur exclusively within the mitochondria of mammals which are activated by increases in $[Ca^{2+}]$ within the same range as pyruvate dehydrogenase, namely NAD^+:isocitrate dehydrogenase and oxoglutarate dehydrogenase (see Denton & McCormack, 1980). All these enzymes are thought to be key sites of regulation of oxidative metabolism and all produce NADH, the principal substrate for ATP production by the respiratory chain.

RR did not affect PDH_a in hearts perfused with control medium in either the presence or the absence of pyruvate, suggesting that in the unstimulated

heart the intramitochondrial [Ca^{2+}] is below the threshold for enzyme activation. Another conclusion suggested by the present work is that Ca^{2+} uptake by mitochondria is not necessary to control cytoplasmic [Ca^{2+}] after an inotropic stimulus, at least in the short term, as both stimulated contraction and phosphorylase *a* content were unaffected by RR. Likewise, it is unlikely that mitochondrial Ca^{2+} is a store for the Ca^{2+} required to stimulate contraction. Moreover, the studies by Peng *et al.* (1980) and Ferrari *et al.* (1982), in pig and rabbit heart respectively, which showed that mitochondrial function could be better preserved during post-ischaemic reperfusion if RR was present, suggest that mitochondria may become damaged if they are exposed to, and allowed to accumulate, excessive amounts of Ca^{2+} (see Denton *et al.*, 1980).

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References

Cohen, P. (1978) *Curr. Top. Cell. Regul.* **14**, 117–196
Denton, R. M. & McCormack, J. G. (1980) *FEBS Lett.* **119**, 1–8
Denton, R. M., Randle, P. J. & Martin, B. R. (1972) *Biochem. J.* **128**, 161–163
Denton, R. M., McCormack, J. G. & Edgell, N. J. (1980) *Biochem. J.* **190**, 107–117
England, P. J. (1976) *Biochem. J.* **160**, 295–304
Ferrari, R., diLisa, F., Raddino, R. & Visioli, O. (1982) *J. Mol. Cell. Cardiol.* **14**, 737–740
Fleckenstein, A. (1977) *Annu. Rev. Pharmacol. Toxicol.* **17**, 149–166
Hiraoka, T., DeBuysere, M. & Olson, M. S. (1980) *J. Biol. Chem.* **255**, 7604–7609
Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Pask, H. T. & Denton, R. M. (1976) *Biochem. J.* **154**, 327–348
Krebs, H. A. & Henseleit, K. (1932) *Hoppe-Seyler's Z. Physiol. Chem.* **210**, 33–66
Luft, J. H. (1971) *Anat. Rec.* **171**, 347–368
Marban, E., Rink, T. J., Tsein, R. W. & Tsein, R. Y. (1980) *Nature (London)* **286**, 845–850
McCormack, J. G. & Denton, R. M. (1981) *Biochem. J.* **194**, 639–643
McCormack, J. G., Edgell, N. J. & Denton, R. M. (1982) *Biochem. J.* **202**, 419–427
Moore, C. L. (1971) *Biochem. Biophys. Res. Commun.* **42**, 298–305
Peng, C. F., Kane, J. J., Straub, K. D. & Murphy, M. L. (1980) *J. Cardiovasc. Pharmacol.* **2**, 45–54
Vasington, F. D., Gazzotti, P., Tiozzo, R. & Carafoli, E. (1972) *Biochim. Biophys. Acta* **256**, 43–54
Whitehouse, S., Cooper, R. H. & Randle, P. J. (1974) *Biochem. J.* **141**, 761–774
Williamson, J. R. (1975) *Handb. Physiol. Sect. 7* **6**, 605–636